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FOREWORD

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PI - Signature

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## I. INTRODUCTION

Tumorigenesis is classically divided into two steps, initiation and promotion. Initiation is likely to be the result of some genotoxic insult. Tumor promotion depends on increased growth of the initiated cell and it may be the result of another genotoxic insult to growth regulatory machinery or it may derive from epigenetic stimuli that are normally involved in regulating growth of the original target tissue. Because of its role in mammary gland development, estrogen has long been suspected to behave as a tumor promoter of mammary cancer. This notion is supported by the fact that mammary tumor growth may be inhibited by antiestrogens. It follows that xenoestrogens could supply tumor promoter activity to initiated mammary cells. The studies devised under this grant are designed to examine the tumor promoter activity of two xenoestrogens, op-DDT and  $\beta$ -hexachlorocyclohexane ( $\beta$ -HCH), to define the role of fat stores of these compounds in mammary tumorigenesis, and to determine the molecular mechanism of estrogenic action of op-DDT and  $\beta$ -HCH.

The body of this report will be divided into sections according to research models studied: 1) the rat methylnitrososurea (MNU)-induced mammary tumor model; 2) human mammary tumor xenograft in athymic mice and standard estrogenic endpoints in mouse reproductive tract tissue; 3) a gene transfection model using estrogen receptor expression vectors and an estrogen-responsive reporter gene. In addition, gas chromatographic analysis of  $\beta$ -HCH and op-DDT in tissue and blood samples from the *in vivo* models has been established and utilized to determine the dose-response parameters required for interpretation of data generated in the present studies and to evaluate data from the literature in relation to the potential for estrogenic effects in humans. The three model systems will be dealt with separately; the description of the analysis of  $\beta$ -HCH and op-DDT in blood and fat is described in the report of work in the mouse model system. An additional section is allotted to a description of some secondary observations made concerning the protective effect of estrogens in cataractogenesis induced by MNU in ovariectomized rats. The Results and Discussion subsection of each report will deal with the scientific significance of the observations made to date, while a separate section will be allotted to the discussion of the relevance to the original Statement of Work and to any adjustments to the research plan.

## II. BODY OF REPORT

### 1. MNU-INDUCED MAMMARY TUMORS IN THE RAT

#### A. BACKGROUND & INTRODUCTION

MNU induces estrogen-dependent mammary tumors in the rat and this has become a standard model system for testing tumor growth inhibiting activity of potential estrogen antagonists. Since injected MNU is fully oxidized and disappears from the animal's system within a matter of less than one hour (1-3), it must produce its tumor initiating effects within minutes of administration. Fully mature, young rats (age, 49-56 days) have been shown to respond optimally to the tumorigenic effect of MNU. Estrogen-dependence of MNU-induced tumors is demonstrable by the following observations: ovariectomy or antiestrogen treatment performed after MNU initiation blocks tumor development; ovariectomy leads to tumor stasis or shrinkage and estrogen replacement will cause resumption of tumor growth. In other model systems the xenoestrogens, op-DDT and  $\beta$ -HCH have been shown to support growth of experimental mammary tumors and/or human breast cancer cells in culture (4, 5). These observations suggest that estrogen is the ovarian factor that is required for tumor progression and that xenoestrogens can substitute for the natural hormone in this regard. However, the tumor promoter activity of estrogens has not been tested directly in the standard MNU rat model.

A study was designed to address the question of whether estrogen behaves as a tumor promoter in the MNU-initiated rat model. There is only one prior report in which MNU-treated animals were ovariectomized before detectable tumors were formed and then treated with estrogen to promote tumorigenesis (6). That study was performed in an unorthodox protocol in which neonatal (2-day-old) rats were treated with MNU, followed by tamoxifen treatment until 30 days of age, at which time animals were ovariectomized, and then estradiol treatment was begun at 3.5 months of age. In this protocol, 50% (6/12) estrogen reconstituted animals developed tumors compared to rates of 80% (35/44) in ovary-intact control animals and 4% (1/27) in ovariectomized, untreated, MNU-initiated rats. Thus, from this single study, it would appear that estrogen can act as tumor promoter in an MNU-initiated mammary gland and therefore estrogen-replacement in ovariectomized MNU-initiated animals should serve as the positive control for the xenoestrogen studies. In our study, animals (49-56 days old) were ovariectomized at the time of MNU treatment and either supplied with a continuous release estrogen capsule or received no further treatment. The notion that estrogen serves a tumor promoter in the mammary gland will be directly tested in this manner. In addition, this model will serve as a test of whether xenoestrogen treatment of ovariectomized rats will, in like fashion, be sufficient to insure tumor progression. The experiments completed to date tested the tumor promoter activity of estradiol and estrone, the latter being a weaker estrogen that predominates in postmenopausal women and which may be more comparable to the weakly estrogenic xenobiotics. Experiments with xenoestrogens are underway but it is too early to report on these at this date.

## B. METHODS

Sprague-Dawley rats (49-56 days old) were ovariectomized and treated with 50 mg/kg MNU (Sigma Chemical Co., St. Louis, MO) by tail vein injection while still under anesthesia (Ketamine). At the time of ovariectomy, a treatment capsule was implanted subcutaneously in each animal. The capsules were made from Silastic tubing (Konigsberg Instruments, Pasadena, CA, 0.062 in. I.D. X 0.125 in. O.D. X 1.4 cm length), sealed at each end with Silastic cement and containing 26-30 mg of crystalline estradiol or estrone, or in the case of the negative control group, the implants remained empty. As a positive control, animals were left intact and implanted with empty Silastic capsules. Beginning at 4 weeks after treatment and continuing through 40 weeks, animals were palpated on a weekly basis to determine the time at which tumors appeared in each animal. The average tumor latency, the time from MNU treatment to detection of the first tumor in each animal, was calculated for each treatment group.

At the end of the experiment (40 weeks), animals were killed by cervical dislocation. Whole body, pituitary, and uterine weights were recorded; tumors were collected and processed for histologic examination. Each animal was skinned to allow visualization of the mammary glands to determine if small tumors had escaped detection during palpation. Mammary tumors were dissected out and processed for histologic examination.

Mammary glands from estradiol-treated, ovariectomized animals were examined by the whole mount technique when the animals were terminated at 8 months. Additional intact animals or ovariectomized, estrone-treated animals were sacrificed at 3 months after initiation of treatment in order to examine the mammary glands. The skins from these animals, with mammary fat pads intact, were immersed in Teliensky's fixative overnight. The mammary fat pads were removed and processed for whole mount observations. Briefly, the fat pads were immersed in graded ethanol solutions (70%, 95%, 100%) for 45 min. each and then defatted by immersion in an acetone bath with 2 changes of 1 h duration and then overnight in fresh acetone. The tissue was then rehydrated and stained in 0.1% toluidine blue for 2 h. The fat pads were destained in methanol and 70% ethanol for 30 min. each and washed in water before immersion in 4% ammonium molybdate for 30 min. to fix the stain. The fat pads were then cleared by dehydration through graded alcohols and xylene. The lightly stained glands were viewed under a dissecting microscope that was interfaced with a Macintosh PowerPC computer (Cupertino, CA) through a Sony CCD video camera; using lymph nodes as landmarks, a standard region of an inguinal or a thoracic gland was imaged.

Tumor incidence was analyzed by Chi-square analysis. All other parameters were analyzed by ANOVA, followed by Fisher's PLSD to test for differences between individual treatment groups.

### C. RESULTS & DISCUSSION

Figure 1 shows the time course of tumor development in the treated animals. By 5 months after MNU treatment, all of the 10 ovary intact animals had palpable mammary tumors; 2 of these animals had tumors as early as 2 months after MNU. Tumors were not detected in estrone- or estradiol-treated, ovariectomized animals until 3.5 or 4 months after MNU, respectively. Average tumor latency for intact controls was 3.4 months while estrone-treated or estradiol-treated animals had average latencies of 4.5 and 4.8 months, respectively (Table 1). (Because of wide variations in this parameter, none of the latency values were significantly different from each other.) At the end of the 40 week observation period, 55% (6/11) of estrone-treated and 24% (5/21) of estradiol-treated animals had tumors (Table 1). The ovariectomized, MNU-treated controls produced no tumors.

Uterine weight, pituitary weight, and whole body weight were determined as measures of the degree of estrogenic stimulation (Table 1). Uterine weights were increased to a similar extent by treatment with either estradiol or estrone compared to the ovariectomized control group, and final uterine weights of the estrogen treated animals did not differ from those of intact control animals. On the other hand, pituitary weights and body weights clearly showed that the estradiol treatment produced excessive estrogenic stimulation, i.e. there was retardation of body weight gain and excessive pituitary weight compared to intact or estrone-treated animals. Such observations support the notion that treatment with an estradiol capsule produced pharmacologic effects while the estrone treatment produced a nearly normal estrogen milieu.

Examination of mammary glands by whole mount preparation showed that estrogen treatments induced a growth response in the tissue (Fig. 2). Mammary glands of intact control animals had an abundance of end buds decorating the ducts, while the ducts of ovariectomized animals were straight and had very few end buds. Treatment with estrone or estradiol induced growth of the mammary tree such that the ducts were decorated with end buds, but not the extent of the normal gland. However, in one of the estrone-treated, ovariectomized animals sacrificed at 3 months of treatment, the thoracic glands appeared much like that of the intact animal (Fig. 2).

Clearly, estrogens produced a tumor promoter effect in our model system. However, in light of the obvious growth response that the estrogen treatments produced, the lack of a full tumor promoter activity seemingly poses a paradox. It may be that there is an optimum dose of estrogen that has not been achieved. From these incomplete data comparing estradiol against estrone, it looks as if a weaker estrogen may have more of a tumor promoting effect; it can be reasoned that administration of a weaker hormone is essentially the same as lowering the dose of hormone. Earlier studies have shown that large doses of estrogen will protect against tumor formation in MNU-treated rats (7); furthermore, it was recently suggested by Guzman et al (8) that this effect is dependent upon a sufficient dose of estradiol to attain pregnancy levels of hormone in the blood serum (ca. 150 pg/ml)

for only one week. It may be that the low incidence of tumors in our estradiol-treated group can be attributed to this protective effect. We had estimated that our Silastic capsules release approximately 1 µg estradiol/day (9); at the end of 40 weeks, serum estradiol was 25-30 pg/ml in estradiol-treated animals and 18-24 pg/ml in estrone-treated animals (estradiol assays were performed in the lab of Dr. Clinton Grubbs, University of Alabama). [At this time we do not have interim blood samples for comparison, however, additional animals are being treated for this purpose]. The data reported by Guzman et al (8) not only showed that a high dose of estradiol was effective at reducing tumor incidence in MNU-treated rats but a dose of one-tenth that which produced the pregnancy levels of estradiol in blood was similarly effective and a further 10-fold reduction of dose still produced some protective effect. Thus, estrogen can have both a protective effect and a promoting effect on mammary gland tumorigenesis. The biphasic nature of the hormone's effect in this regard is not atypical of hormone action in general but it requires further investigation to determine the boundary between these two opposing responses.

## **2. MOUSE MODEL – MCF-7 XENOGRAFTS & REPRODUCTIVE TRACT RESPONSES**

### **A. BACKGROUND & INTRODUCTION**

The xenograft of human tumor cells in athymic mice has become another standard model for testing the tumor growth promoting or inhibiting effects of a test compound. We had shown earlier that  $\beta$ -HCH stimulated growth of tumors produced from the human breast cancer cell line, MCF-7 (5). In that study, host animals were treated with a single Silastic capsule containing either  $\beta$ -HCH or estradiol; tumor growth was essentially equivalent for both treatments over a 16 day period. The goal of this part of the proposed studies was to test the effects of dietary restriction on this growth promoting effect of  $\beta$ -HCH or op-DDT but it was reasoned that the model system required definition of dose-response parameters before embarking on the ultimate experiment.

In a preliminary study we sought to determine the blood and tissue levels attained following implantation of Silastic capsules containing either  $\beta$ -HCH or op-DDT and to relate these levels to the biological response in the host animals. This preliminary study is partially completed and has yielded useful dose-response information. We have also performed a single xenograft experiment based on that information, using the highest dose of test compound. Paradoxically, neither op-DDT nor  $\beta$ -HCH promoted growth of the MCF-7 tumors in this experiment; this may be due to a change in the xenografting protocol or to a biphasic dose-response such as we suspect in the case of the rat MNU model system (see Discussion).

## B. METHODS

### Dose-Response of *op*-DDT and $\beta$ -HCH administered to mice.

*Animal treatment:* Silastic capsules were made as described above with the exception that the length of capsule containing crystalline compound was 0.8 cm so that each capsule contained 18-22 mg of material. Adult ICR mice were ovariectomized and 3 weeks later groups of five animals received 1, 2, or 4 treatment capsules containing either *op*-DDT or  $\beta$ -HCH. A positive control group consisted of animals that received a Silastic capsule containing estrone (Sigma) and negative control animals were treated with an empty Silastic capsule. After 1 week of treatment, animals were anesthetized and exsanguinated by heart puncture. The uterus and vagina of each animal was processed for histomorphometric determination of the estrogenic effects; blood serum and intraperitoneal fat samples were analyzed for *op*-DDT or  $\beta$ -HCH content by gas chromatography.

*Histomorphometrics:* Pieces of uterus and vagina were fixed in neutral formalin and processed for paraffin sections (6  $\mu$ m). Tissue sections were stained with hematoxylin and eosin. Cross-sections were examined under a light microscope (Nikon Optiphot) that was interfaced with a Macintosh PowerPC computer (Apple, Cupertino, CA) through a Sony 3CCD color video camera. The height of the uterine epithelium and the thickness of the vaginal epithelium were determined using an image analysis program (IPLab Spectrum, Signal Analytics, Vienna, VA).

*Tissue Extractions & Gas Chromatography:* Approximately 100 ml mouse serum was extracted three times with 20 ml of hexane (EM Scientific, Gibbstown, NJ). In order to increase the volume of the aqueous phase, 1 ml of 90% formic acid (Fisher Scientific, Fair Lawn, NJ) was added to the serum. The internal standard was  $\gamma$ -HCH for the mice treated with  $\beta$ -HCH and *pp*-DDT was used for the mice treated with *op*-DDT (Accustandard, New Haven, CT). The resulting aqueous phase was extracted with 1 min of vortex mixing and 1 min of centrifugation for each aliquot of hexane.

Fat tissues (about 0.25 g) were ground with about 15 g of 10-60 mesh anhydrous sodium sulfate (Fisher Scientific) and spiked with the appropriate internal standard as above. These mixtures were then soxhlet extracted for 24 hours in 300 ml of 50% acetone in hexane (EM Scientific).

With each set of 6 samples, a matrix spike and either a matrix blank or a glassware blank were also extracted. All of these quality control samples underwent the same cleanup procedure as the samples. The matrix spike contained a known amount of the target analyte similar in concentration to what was expected in the samples. No target compound was found in any type of blank; therefore, blank correction was not necessary.

Lipid analysis was performed gravimetrically in duplicate. The removal of lipids was performed using a gel permeation chromatography column. The glass column (2.5 cm x 100 cm) was packed with SX8 Bio-Beads (BioRad Laboratories, Hercules, CA) and eluted with 60% cyclohexane in dichloromethane (EM Scientific) at a flow

rate of 10 ml/min through the column. The lipids were eluted in the first 20 min fraction, and the HCHs and DDTs were eluted in the following 40 min fraction.

Sample extracts were reduced to about 1 ml by rotary evaporation and exchanged into hexane as necessary. These extracts were then run through a silica (grade 923 Grace Davison, Baltimore, MD) column consisting of glass wool, 20 cm of silica (1% HPLC grade water deactivated), and 1 cm of sodium sulfate. Three fractions were collected, consisting of 75 ml each hexane, 50% dichloromethane in hexane for the HCH analysis or 20% for the DDT analysis, and dichloromethane with a 10 ml switching volume. The HCHs and DDTs were in the second fraction. All three fractions were rotavapped and solvent exchanged into hexane, if necessary. The second fraction was further reduced to about 50 ml by a stream of nitrogen and transferred into an autosampler vial with 2-3 rinses of hexane.

The samples were analyzed on a Hewlett Packard 5890A gas chromatograph with an electron capture detector. The carrier gas was hydrogen (80 ml/min) and the makeup gas (25 ml/min) was nitrogen (Gas Tech, Hillside, IL). Injections were made by an autosampler in the splitless mode, and the purge flow (2 ml/min) was opened after 3 min. A 60 m DB5 column (J&W Scientific, Folsom, CA) with an internal diameter of 250 mm and a film thickness of 0.1 mm was used for separation. The temperature program for the DDTs was 40°C for 1 min, 30°C/min to 130°C, 3°C/min to 241°C, 30°C/min to 285°C with a 10 min hold for a total analysis time of 52.46 min. The HCH temperature program was 50°C for 1 min, 20°C/min to 130°C, 1 °C/min to 160°C, 30°C/min to 290°C, with a 1 min hold for a total analysis time of 40.33 min.

Relative response factors were used to quantify the concentrations of analytes. Integrated peak areas of known concentrations were then compared to the samples by the following equations. A = integrated peak area, m = mass (ng)

$$RRF = (m_{\text{analyte}}/A_{\text{analyte}})/(m_{\text{standard}}/A_{\text{standard}})$$

$$m_{\text{analyte}} = A_{\text{analyte}} * RRF * (m_{\text{standard}}/A_{\text{standard}})$$

#### MCF-7 Xenograft Experiment

Host animals (5-6 week old, athymic, Balb/c mice, were ovariectomized and implanted with a single Silastic capsule containing estradiol. MCF-7 cells ( $10^9$ ) were harvested from cell cultures and suspended in 5 ml medium (MEM, supplemented with 5% FBS). Matrigel (0.75 ml, Collaborative Research Products, Cambridge, MA) was added to the cell suspension. Host mice (16 total) received 100  $\mu$ l injections (approximately  $1.7 \times 10^7$  cells) of this cell suspension into the left and right thoracic mammary fat pads. After 3 weeks the estradiol capsules were removed from 3 groups of 4 animals and 2 of these groups were treated by inserting 4 capsules of either op-DDT or  $\beta$ -HCH into each animal. At this time and at weekly intervals

thereafter, tumors were measured with calipers and tumor volumes were determined, as described earlier <sup>5</sup>.

### C. RESULTS AND DISCUSSION

#### *Mouse reproductive tract responses and xenoestrogen blood levels*

Animals treated with 1, 2, or 4 capsules of  $\beta$ -HCH or op-DDT showed linear increases in blood and fat concentrations of those compounds (Figs. 3 & 5). It is apparent from these linear increases that saturation of the tissues has not been reached, even at the highest dose applied. Macholz et al (10) showed that feeding rats food containing either 600 or 3000 ppm  $\beta$ -HCH produced concentrations in the fat of 250  $\mu\text{g/g}$  or 210  $\mu\text{g/g}$ , respectively, at 30 days, indicating that the fat was saturated. The highest level achieved in our study was 55  $\mu\text{g}$   $\beta$ -HCH/g fat tissue at the end of one week of treatment. In an earlier study, Dale et al (11) reported that feeding rats 200 ppm of DDT for 90 days produced fat levels of 729-2206  $\mu\text{g/g}$  in female rats. In our study, after one week with 4 treatment capsules the fat levels of op-DDT were 56-99  $\mu\text{g/g}$ .

The lowest dose of compound used in this mouse study produced blood and fat levels that were only 1 order of magnitude higher than the highest levels recorded in humans with no history of excess exposure. The lowest dose of op-DDT in our study produce blood levels of 76-276 ng/ml and fat levels of 8.1-12.9  $\mu\text{g/g}$ . Human blood levels of all isomers of DDT and their metabolites are generally 5-30 ng/ml (12). Although the op-isomer is only a very small proportion of total DDT isomers contaminating the environment, a group of Israeli men were found to have blood levels of op-DDT averaging about 6 ng/ml (12). In the present mouse study, a single capsule of  $\beta$ -HCH produced blood levels of 56-74 ng/ml. Human blood levels of  $\beta$ -HCH are generally 0-5 ng/ml but have been measured as high as 31 ng/ml (12).

Uterine epithelium exhibits both hypertrophic and hyperplastic responses to estrogen stimulation. The hyperplastic response is apparent from the extremely crowded character of the epithelium; although it is a simple type of epithelium, under estrogen stimulation it takes on the appearance of a stratified layer of cells, i.e. it becomes pseudostratified. The hypertrophic response of the epithelial cell is apparent from its increase in cell height. Upon ovariectomy, the columnar epithelium becomes cuboidal with an average height of approximately 8  $\mu\text{m}$ ; stimulation with estrone increased the epithelial height to 16-25  $\mu\text{m}$  (Fig. 4). As seen in figure 5B, op-DDT increased uterine epithelial height to 11-26  $\mu\text{m}$  in a dose dependent fashion. The uterine response to  $\beta$ -HCH was quite different, the lowest dose produce a significant increase in epithelial cell height, to 12  $\mu\text{m}$ , but there was no further increase with increasing dose (Fig. 5D).

Vaginal epithelium undergoes a dramatic increase in cell proliferation in response to estrogenic stimulation. With full stimulation from a strong estrogen the superficial layer of cells loose their nuclei and organelles to become a flattened layer of keratin; continuous stimulation produces multiple laminations within this keratinized superficial layer. In the ovariectomized control animals the epithelium

consisted of a very low layer of squamous cells that was one to two cells thick, approximately 10  $\mu\text{m}$ . Estrone-treated animals had a vaginal epithelium that was multilayered with a fully keratinized surface layer; the epithelial thickness, below the keratinized layer, was 78-97  $\mu\text{m}$ . Thickness of the vaginal epithelium was increased in a dose-dependent manner after treatment with either op-DDT or  $\beta$ -HCH (Fig. 5A & C). All of the animals treated with op-DDT, including the lowest dose, had fully keratinized vaginal epithelia. Although the vaginal epithelium of all the animals treated with  $\beta$ -HCH showed stimulation, only two of the five animals with four treatment capsules exhibited a keratinized epithelium and none of the others had a keratinized surface layer. Thus there appears to be both a quantitative and a qualitative difference between the estrogenic characters of  $\beta$ -HCH and op-DDT.

The object of these dose-response studies was to determine the lowest dose of compound that would produce an estrogenic effect and to correlate tissue levels against the estrogenic effect. This would aid in completion of our present studies in that knowing the effective blood and tissue levels will help in the interpretation of the data on tumor growth in both the rat model (above) or in the mouse xenograft model. It would also be useful for comparing known human blood and tissue levels against putative estrogenic effects. Without further study it is not possible to define the minimal level of compound required for estrogenic effects in mice. The lowest doses of both compounds used in the studies reported here were effective in stimulating vaginal epithelial proliferation and increased cell height in the uterine epithelium. The blood levels achieved in the test animals by these low doses of  $\beta$ -HCH and op-DDT are within 1 order of magnitude of the values for these compounds in some of human samples. Further study with even lower doses is required to identify tissue levels that are without estrogenic effect.

#### MCF-7 tumor growth

In an earlier report we showed that  $\beta$ -HCH was able to stimulate growth of MCF-7 cells grown as tumors in xenograft on athymic mice (5). In that study the cells had been passaged from one xenograft into the test animals that were included in the experiment. This procedure of passaging tumor tissue into experimental animals was found to produce measurable tumors with very little lag time; furthermore, the xenografted tumor tissue grew in ovary intact athymic mice, without need for estrogen administration. We have had difficulty in trying to set up a replication of that original experiment. First, we have had difficulty in re-establishing a xenograft tumor line of MCF-7 cells. Eventually we found that suspending the cells in Matrigel and implanting an estradiol capsule in the prospective host allowed us to generate estrogen-dependent tumors. [Originally, we had established the xenografts by injecting cells suspended only in culture medium; there is no explanation of why this did not work again.] Use of the Matrigel produced measurable tumors in just 3 weeks if the hosts were under estrogen stimulation from a Silastic implant; since this was the case, we have used this system for setting up an experiment rather than passaging tumor tissue from one animal into others. It was reasoned that the

suspended cells would produce more consistent size of tumor as the number of cells injected into each site could be better controlled.

Host animals were ovariectomized and received a Silastic implant containing estradiol. One week later, each animal received two injections of MCF-7 cells suspended in Matrigel; the cells were injected into the thoracic mammary fat pads just posterior to the front legs. At 3 weeks, animals were anesthetized and the estradiol capsules were removed and replaced with 4 capsules containing either op-DDT or  $\beta$ -HCH, or with 1 empty capsule. One group of animals was left with its estradiol capsule in place. At this time, each tumor was measured with a pair of calipers and the volume of each tumor was calculated.

As seen in Fig. 6, tumors continued to grow in animals that were maintained on estradiol treatment, while tumors regressed in all three of the other treatment groups. Thus, administration of op-DDT or  $\beta$ -HCH in doses that are known to be estrogenic in the vaginal and uterine epithelium was ineffective in maintaining the growth of the MCF-7 tumors. The reason for the difference in these results and those of our earlier, published results requires further investigation. It may be that the tumor that was passaged from animal to animal in our earlier study was more sensitive to estrogens. In support of this it can be seen that the growth response to estradiol of the passaged xenograft was much more robust than that of the tumor formed from implanted cells; the original tumor increased in volume 6-fold over a 16 day period of estradiol stimulation while the volume of estradiol treated tumor in the present study increased 4-fold over a 42 day period. In addition, the levels of endogenous hormone produced by the host's ovaries proved sufficient to maintain growth in the original study while, in preparing for this study, we could not maintain the freshly formed tumors without supplemental hormone administration. Additional studies will be required to compare tumors formed through the two protocols.

### **3. ESTROGEN RECEPTOR-REPORTER GENE TRANSFECTION STUDIES**

#### **A. BACKGROUND & INTRODUCTION**

Estrogens produce a variety of biological effects in different tissues: they stimulate growth of the uterus and mammary gland cells, maintain bone mineral density, and reduce serum LDL (13, 14). Recently, it has been appreciated that certain compounds selectively modulate these biological effects; for instance, tamoxifen behaves as an antiestrogen in mammary carcinoma cells but is stimulatory in the uterus and bone (14). The divergent effects of compounds such as tamoxifen has led to the hypothesis that there are substance that can act as selective estrogen response modulators (SERMs) (14). Some xenoestrogens appear to behave as SERMs, modifying biological responses in some tissues but not in others (13). The mechanisms by which SERMs specify tissue response is unknown.

The estrogen receptor protein can be divided into four functional, and physically distinct, domains: ligand binding domain; the DNA binding domain; the N-terminal transcriptional activation function domain (AF-1); and the C-terminal transcriptional activation function domain (AF-2) (15-18). AF-1 and AF-2 behave differently depending the ligand present, the cellular context, and the gene promoter under activation (18, 19). It may be that additive or synergistic effects of the two activation domains are regulated differentially by SERMs.

Steroid receptors do not activate gene transcription on their own, rather they elicit this function by binding coactivator proteins and thereby generating and interacting with a large complex of transcription factors (20). The coactivator proteins, SRC-1, TIF2 and GRIP-1, interact with the estrogen receptor mainly at the AF-2 region (20-23). However, recently it has been shown that coactivators can act through the AF-1 region as well (24, 25). This interaction at two sites within the protein may allow for the additive or synergistic actions required to explain the function of SERMs.

The three-dimensional structure of the estrogen receptor has been partially deciphered by examining the crystal structure of the carboxy half of the protein in the presence or absence of ligand (26, 27). It was found that compounds with antiestrogen activity, such as tamoxifen and raloxifene, produce a conformation of receptor that would preclude its interaction with coactivator in the AF-2 region, while estradiol produces a conformation that promotes this interaction (26, 27). Although this suggests a mechanism for the antiestrogenic effects of tamoxifen or raloxifene, it does not explain how they can behave as estrogen agonists in certain tissues.

The present studies were designed to dissect the ability of xenoestrogens to function through the different domains of the estrogen receptor. Mutant receptors lacking either AF-1 or AF-2 were used in transfection assays and the transactivational effects of the compounds were compared against that of estradiol. The results suggest that xenoestrogens are more dependent upon the interaction of the two functional domains than is estradiol and that this is especially true for particular compounds.

## B. MATERIALS AND METHODS

*Reagents:* Cell culture reagents (phenol-red free MEM, glutamine, phosphate buffered saline calcium and magnesium free, and DMRIE-C) were purchased from Life Technologies. Fetal calf serum and charcoal-stripped fetal calf serum were purchased from Hyclone Laboratories. Estrogen, bisphenol A, and octylphenol were purchased from Sigma.  $\beta$ -HCH and o,p'-DDT and were purchased from Chemservice. Luciferase assay reagents were purchased from Promega while the  $\beta$ -galactosidase assay reagents were purchased from Tropix.

*Plasmid construction:* Wild-type ER $\alpha$  expression plasmid ER $\alpha$ pCMV5 (28), parental pCMV5 plasmid, R507L (29), S118A (30), and ER $\alpha$  $\Delta$ AB (30) were provided by Benita Katzenellenbogen. Mutant ER $\alpha$  expression plasmids HE11 and HE48 (15) were provided by Pierre Chambon. Mutant ER $\alpha$  expression plasmids TAF1 and ER-Null (18) were provided by Donald McDonnell. The  $\beta$ -galactosidase expression plasmid pAD was purchased from Stratagene. The parental luciferase reporter plasmid pGL3 was purchased from Promega.

The reporter plasmid was constructed as follows. First, the minimal promoter region of the pS2 gene (15, 31, 32) was synthesized by hybridizing the oligonucleotides:

GATCCCCGGGCCTCTAGGCAAATGTTATCTAACGCTCTTAAGCAAACAGAGCC  
TGCCCTATAAAATCCGGGGCTCGGGCGGCCTCTCATCCCTGACTTCGA

and

AGTCAGGGATGAGAGGCCGCCGAGCCCCGGATTTATAGGGCAGGCTCTGTTGCT  
TAAAGAGCGTAGATAAACATTGCCTAAGGAGGCCGGG.

The DNA was blunted with Klenow fragment and ligated into the blunted HindIII site of pGL3; this plasmid is referred to as pS2Luc. To make this reporter estrogen responsive, we synthesized an enhancer region containing two estrogen response elements (EREs, underlined in the sequence below) by hybridizing the following oligonucleotides:

CAGGTCACAGTGACCTGATCAGCTAGTCCAGGTCACAGTGACCTCGTAC

and

GAAGGTCACTGTGACCTGACTAGCTGATCAGGTCACTGTGACCTGGTAC.

This DNA was blunted with Klenow fragment and ligated into the blunted KpnI site of pS2Luc to make ERE2pS2Luc.

The estrogen receptor mutant containing an AF-1 deletion, ER $\alpha$  $\Delta$ 2-150, was constructed by amplifying a PCR fragment from ER $\alpha$ pCMV5 under the manufacturers suggested conditions using primers:

GCCCAGGGGCCGCTCCAGGGGATC and CATGGTCCGTGGCCGCGGGCAGGGT and rTth DNA polymerase XL (Perkin-Elmer). The PCR fragment was circularized with T4 ligase (Gibco) and treated with DpnI. Then, the DNA was used to transform DH5 $\alpha$  bacterial cells. The ERE2pS2Luc and ER $\alpha$  $\Delta$ 2-150 plasmids were confirmed by dideoxysequencing.

*Cell culture and Transfection:* MDA-MB-231 breast cancer cells (ATCC) were maintained in phenol-red free MEM containing 2 mM glutamine and 5% fetal calf serum. In preparation for transfection, cells were plated at a density of 200,000 cells per 35 mm well in phenol-red free MEM containing 2 mM glutamine and 5% charcoal-stripped fetal calf serum. The next day cells were transiently transfected using DNA-DMRIE-C lipid complexes. Briefly, 500 ng of ERE2pS2Luc reporter plasmid, 100 ng of pAD  $\beta$ -galactosidase expression plasmid, and 25 ng estrogen receptor expression plasmid were incubated for 30 minutes with 1 ml of phenol-red

free MEM and 4  $\mu$ l of DMRIE-C reagent. DNA-lipid complexes (1 ml) were placed on each well of MDA-MB-231 cells for 6 h.

The transfected cells were treated with various concentrations of estradiol, bisphenol A, octylphenol, o,p'-DDT,  $\beta$ -HCH, and DMSO for 24h in medium supplemented with 5% charcoal-stripped serum. Cells were washed once with phosphate buffered saline calcium and magnesium free and lysed with 200  $\mu$ l of reporter lysis buffer (Promega). Lysate was exposed to one freeze-thaw cycle and then centrifuged. Briefly, 80  $\mu$ l of luciferase reagent was mixed with 20  $\mu$ l of lysate and light output was measured with a ML3000 luminometer. A separate 20  $\mu$ l sample was analyzed for  $\beta$ -galactosidase activity by mixing 80  $\mu$ l of assay buffer and incubating at room temperature for 1 h. Then, 100  $\mu$ l of light accelerator was added and light output was measured. The  $\beta$ -galactosidase measurements normalized the observed luciferase activity.

### C. RESULTS & DISCUSSION

Since certain environmental estrogens show tissue-selective effects in the ovariectomized rat (13), we tested the hypothesis that these compounds produce distinct changes in transcriptional efficiency of the estrogen receptor when compared to estradiol and that the relative contribution of the two activation function domains within the receptor would be ligand specific. To measure the transcriptional effect of these environmental estrogens, we developed a co-transfection assay. The assay allowed us to introduce wild-type estrogen receptor cDNA as well as specific functional mutants of the receptor into an estrogen receptor negative cell line, MDA-MB-231. Using a luciferase reporter plasmid that contained two consensus estrogen response elements upstream of a minimal pS2 promoter, ERE2pS2Luc, we measured the transcriptional activity of the estrogen receptor. The amount of luciferase produced by the transfected MDA-MB-231 cells is dependent on the presence of estrogen receptor and concentration of compound placed on the cells during culture. A hormone-independent  $\beta$ -galactosidase expression vector was cotransfected into the cells and the  $\beta$ -galactosidase activity was used to normalize for well-to-well variation in transfection efficiency. Maximal luciferase activity at  $10^{-9}$  M estradiol was defined as 100% activity and was used to determine the relative transcriptional activity of compounds for each estrogen receptor expression plasmid tested.

*Wild-type estrogen receptor:* To measure the activity of the environmental estrogens, bisphenol A, octylphenol, o,p'-DDT, and  $\beta$ -HCH, we introduced wild-type estrogen receptor, ER $\alpha$ pCMV5, into MDA-MB-231 cells. With  $10^{-10}$  M estradiol, the luciferase activity reached maximal levels producing a five-fold increase relative to DMSO control (Fig. 7). The half-maximal concentration of estradiol ( $K_d$ ) was approximately  $1.0 \cdot 10^{-11}$  M (Fig. 7), similar to that found earlier by Reese and Katzenellenbogen (28).

Since our co-transfection assay was sensitive enough to detect transcriptional activation by a subnanomolar concentration of estrogen, we could measure increases in luciferase activity induced by weak binding environmental estrogens. With bisphenol A and octylphenol, the luciferase activity increased about five-fold relative to DMSO control (Fig. 7). So the environmental estrogens, bisphenol A and octylphenol, activate the wild-type estrogen receptor with the same efficacy as estradiol. The concentration of bisphenol A and octylphenol required to reach maximal luciferase activity was  $10^{-6}$  M. The observed  $K_d$  for bisphenol A was approximately  $0.3 \cdot 10^{-6}$  M whereas the  $K_d$  for octylphenol was approximately  $1.0 \cdot 10^{-7}$  M (Fig. 7). So, although bisphenol A and octylphenol activate the estrogen receptor as strongly as estrogen, the compounds have a four-log reduced potency reflecting their weak binding affinity for the estrogen receptor.

The environmental estrogen, o,p'-DDT, showed an interesting induction of luciferase activity compared to estradiol. The maximal luciferase activity by  $10^{-5}$  M o,p'-DDT was about seven-fold compared to DMSO control (Fig. 7). However, the concentration of o,p'-DDT required to reach plateau was not determined because the limits of solubility for o,p'-DDT had been reached at  $10^{-5}$  M. So, the observed maximal transcriptional activity for o,p'-DDT was greater than the transcriptional activity for estradiol. The super-activation of the estrogen receptor by o,p'-DDT may be due to a ligand-independent activation of the estrogen receptor through phosphorylation by kinases activated by high concentrations of o,p'-DDT (33).

$\beta$ -HCH was also tested in our co-transfection assay. Although  $\beta$ -HCH does not displace tritiated estradiol from receptor (5), it does activate luciferase expression in our system. Luciferase activity is increased about four-fold compared to DMSO control (Fig. 7). Again, similar to o,p'-DDT,  $\beta$ -HCH did not show a plateau of activity at the highest concentration tested. However, unlike o,p'-DDT,  $\beta$ -HCH did not show a super-activation of the wild-type estrogen receptor (Fig. 7).

Estrogen receptor with loss of AF-1 function. To measure the activity of the environmental estrogens on an estrogen receptor without an AF-1 domain, we introduced the ER $\alpha\Delta 2$ -150 or ER $\alpha\Delta AB$  plasmids into MDA-MB-231 cells. Although the absolute amount of luciferase expression was reduced with the ER mutants compared to wild-type ER, the fold response remained five- to eight-fold with the mutants tested (Fig 8, 9). For example, estradiol increased the luciferase activity about five-fold relative to DMSO control (Fig. 8, 9). Furthermore, the  $K_d$  for estradiol using the AF-1 defective mutants was similar to the  $K_d$  for the wild-type receptor (Fig 7-9). The literature shows the binding affinity for AF-1 mutant receptors does not differ from the wild-type receptor. Estradiol produced maximal luciferase activity at  $10^{-10}$  M. Similar to estradiol, bisphenol A, octylphenol, o,p'-DDT, and  $\beta$ -HCH had  $K_d$ 's that were identical when comparing between wild-type receptor and the AF-1 defective mutants (Fig 8, 9). The experiment suggests that deleting the AF-1 domain does not reduce the binding affinity of compounds to the hormone binding domain of the estrogen receptor.

Whereas the environmental estrogens activated the wild-type receptor as efficaciously as estradiol, the AF-1 defective estrogen receptors had reduced transcriptional activity. With bisphenol A, the luciferase activity was only 54% of the maximal activity reached by using  $10^{-9}$  M estradiol (Fig. 8). Similarly, with the ER $\alpha\Delta$ AB mutant treated with bisphenol A, luciferase activity reached 45% of maximal estradiol activity (Fig. 9). The transcriptional activity was also reduced for octylphenol, o,p'-DDT, and  $\beta$ -HCH. Octylphenol showed 65% of the maximal estradiol activity (Fig. 8). Also, with the ER $\alpha\Delta$ AB mutant, octylphenol was 84% of maximal estradiol activity (Fig. 9). Like the wild-type estrogen receptor, the transcriptional response of the AF-1 defective mutants was saturated by micromolar concentrations of bisphenol A and octylphenol (Fig. 7-9). In contrast, high concentrations of o,p'-DDT and  $\beta$ -HCH did not saturate the transcriptional response of ER $\alpha\Delta$ 2-150 or ER $\alpha\Delta$ AB (Fig. 7-9). However, the maximal transcriptional response of these two compounds was reduced in ER $\alpha\Delta$ 2-150.  $\beta$ -HCH activated ER $\alpha\Delta$ 2-150 at 67% of the maximal estradiol activity while o,p'-DDT activated at 92% of the maximal estradiol activity (Fig 8). Thus, o,p'-DDT lost its ability to super-activate transcription when the receptor lacked the AF-1. Overall, the transcriptional activity of the AF-1 defective mutants is reduced compared to the wild-type receptor. Moreover, the transcriptional activity induced by environmental estrogens is further reduced compared to estradiol.

The literature shows that the estrogen receptor uses both AF-1 and AF-2 for transcriptional activation (16, 18, 34). Our data agrees with published observations that the reduced transcriptional activity of ER $\alpha\Delta$ 2-150 using estradiol is due to activity from the functional AF-2 domain. However, since the activity from environmental estrogens is further reduced, our data also suggests that there are different ligand-induced conformations of the AF-2 domain. To increase reporter activity, the AF-2 domain interacts with the transcriptional machinery through coactivators such as the SRC-1 family, GRIP1, and TIF2 (20-23). Because the transcriptional activity is reduced with environmental estrogens, it implies that the interaction of AF-2 with coactivators is reduced. The conformation adopted by the hormone binding domain determines the affinity of the estrogen receptor for coactivators. For example, tamoxifen and raloxifene produce conformations in the carboxy portion of the estrogen receptor that cannot interact with coactivators (26, 27). Perhaps the environmental estrogens cause unique conformations of the estrogen receptor so that the AF-2 binds to coactivators with different affinities. The conclusion would be that the transcriptional machinery senses the conformation of the estrogen receptor bound by environmental estrogens. When operating with an isolated, functioning AF-2, the conformation adopted by the receptor bound to an environmental estrogen shows reduced transcriptional activity.

*Estrogen receptor with loss of AF-2 function:* To measure the activity of the environmental estrogens on an estrogen receptor without an AF-2 domain, we introduced the TAF1 plasmid into MDA-MB-231 cells. The TAF1 plasmid has point mutation in the AF-2 region of the receptor that interacts with coactivators (18);

thus, this mutant receptor depends on the AF-1 domain for transactivational function. Similar to ER $\alpha$  $\Delta$ 2-150, the absolute amount of luciferase expression was reduced with TAF1 compared to wild-type ER; however, the response at 10<sup>-9</sup> M estradiol was 10-fold compared to vehicle control (Fig. 10). We observed that the dose-response curves were right-shifted one log compared to the wild-type receptor (Figs. 7 & 10). In contrast, TAF1 has a binding affinity for estradiol similar to wild-type estrogen receptor since the point mutations in TAF1 do not interfere with hormone binding (18). Lower numbers of receptors in the transfected MDA-MB-231 cells may explain the right-shift in the dose-response curves. Other transfection experiments using TAF1 showed dose-response curves similar to wild-type receptor (data not shown).

Each dose-response curve from the environmental estrogens had a one log right-shift when compared to wild-type estrogen receptor (Figs. 7 & 10). Whereas the compounds activated the wild-type receptor as efficaciously as estradiol, this AF-2 defective estrogen receptor had reduced transcriptional activity induced by the environmental estrogens. In fact, the transcriptional activity was reduced to a greater extent compared to the AF-1 defective mutant. With bisphenol A, the luciferase activity was only 27% of the maximal activity by 10<sup>-9</sup> M estradiol (Fig. 10).  $\beta$ -HCH activated TAF1 at 13% of the maximal estradiol activity while o,p'-DDT was at 54% of the maximal estradiol activity (Fig. 10). Octylphenol showed 38% of the maximal estradiol activity (Fig. 10). Although the dose-response curves are right-shifted one log, the transcriptional response of TAF1 was saturated by micromolar concentrations of bisphenol A and octylphenol whereas high concentrations of o,p'-DDT and  $\beta$ -HCH did not saturate the transcriptional activity (Fig. 10).

Our experiments show that the transcriptional activity of the AF-2 defective mutant is reduced compared to wild-type receptor. The data suggest that the reduced transcriptional activity using environmental estrogens compared to estradiol is due to different ligand-induced conformations in the AF-2 defective mutant. To increase reporter activity, the TAF1 receptor interacts with the transcriptional machinery through the functioning AF-1 domain. Because the activity is, in general, reduced with environmental estrogens, it implies that the interaction of AF-1 with the transcriptional machinery is reduced. There is evidence that coactivators interact with AF-1 to produce a transactivational response, but the region of AF-1 that mediates this interaction has not been defined to the extent that it has for the AF-2 region (23). It may be that the transcriptional machinery senses the conformation of the estrogen receptor bound by environmental estrogens, thereby producing the tissue selective activity of certain environmental estrogens.

Estrogen receptor with loss of AF-1 and AF-2 function. To measure the activity of the environmental estrogens on an estrogen receptor without either an AF-1 or AF-2 domain, we introduced ER-Null into MDA-MB-231 cells. For every compound and every concentration tested, the luciferase activity was about 2% (Fig. 11). MDA-MB-231 cells transfected with the parental pCMV5 plasmid produce a

similar amount of luciferase (data not shown). The luciferase activity produced by ER-Null reflects the basal activity of the pS2 minimal promoter. Our data shows that no other cryptic transcription activation functions other than AF-1 and AF-2 are involved in the transcriptional activity of the estrogen receptor in our system (35). Thus, in our co-transfection assay, we are measuring the transcriptional activity of only AF-1 and AF-2 without interference from other transcriptional activation functions present in the receptor.

*Estrogen receptor with mutation of serine 118 to alanine:* Protein growth factors can produce estrogen-like transcriptional effects (36, 37) and the mechanism may be through MAPK kinase phosphorylation of the estrogen receptor at serine 118 (37, 38). We introduced the S118A mutant receptor into MDA-MB-231 cells since S118A is defective in signaling through the MAPK kinase pathway (38). The phosphorylation of S118 by MAPK kinase produces a ligand-independent increase in transcriptional activity of the estrogen receptor. Since high concentrations of certain environmental estrogens activate phosphorylation pathways, we examined whether the transcriptional activity in wild-type estrogen receptor is due to phosphorylation of S118. We observed that the absolute activity of S118A was similar to wild-type estrogen receptor (data not shown). With estrogen, the luciferase activity increased about five-fold relative to DMSO control and the  $K_d$  was approximately  $1.0 \cdot 10^{-11}$  M (Fig. 12) which is similar for the wild-type receptor since S118A binds estradiol with wild-type affinity.

The S118A transcriptional activity induced by environmental estrogens was similar to activity from wild-type estrogen receptor. With bisphenol A, octylphenol, and  $\beta$ -HCH the luciferase activity increased four- to five-fold relative to DMSO control (Fig. 12). The observed  $K_d$  for each of the environmental estrogens was similar for the S118A mutant and the wild-type receptor (Figs. 7 and 12). Similar to wild-type estrogen receptor,  $10^{-5}$  M o,p'-DDT super activated S118A eight-fold compared to DMSO control (Fig. 12). The transcriptional activity was not saturated by high dose o,p'-DDT. Thus, transcriptional activation by estradiol or the xenoestrogens is not dependent upon phosphorylation of serine 118, nor is the super-activation of the receptor by o,p'-DDT due to phosphorylation at serine 118. However, other potential phosphorylation sites are intact in the S118A mutant and other serine mutants must be tested to determine if ligand-independent kinase activation of the estrogen receptor occurs.

*Estrogen receptor with a reduction in hormone binding:* The mutant R507L binds estradiol with lower affinity for estradiol. We asked whether environmental estrogens utilize the same amino acid residue for ligand dependent binding of compounds. In the experiment, we observed that  $10^{-8}$  M estradiol caused maximal transcriptional activity in R507L (Fig. 13). Compared to wild-type estrogen receptor, the dose-response curve of R507L is right-shifted nearly two logs. The R507L receptor had an absolute luciferase activity similar to wild-type estrogen receptor (data not shown). However, since the basal level of transcriptional activity is low,

R507L is activated about 60-fold with estradiol (Fig. 13) giving R507L a large activation 'window'.

When the environmental estrogens were tested, virtually no activity was seen with bisphenol A, octylphenol, o,p'-DDT, or  $\beta$ -HCH (Fig. 13). Two explanations are possible. First, the amino acid 507 may be critical and required for the binding and transcriptional activity of the environmental estrogens tested. Second, a more likely explanation is that the dose-response curves of the environmental estrogens were right-shifted by two logs. If that is the case, then even at  $10^{-5}$  M none of the compounds are at sufficient concentrations to activate R507L. If larger concentrations of compounds could be achieved perhaps activation would be seen. However, concentrations higher than  $10^{-5}$  M could not be tested since all of the compounds have reached their solubility limits. We conclude that amino acid 507 is required for the activity of weak binding environmental estrogens.

*Estrogen receptor with loss of DNA binding activity:* To measure the activity of the environmental estrogens on an estrogen receptor without any DNA binding activity, we introduced HE11 into MDA-MB-231 cells. For every compound and every concentration tested, the amount of luciferase activity measured was about 2% which reflects the basal activity of the pS2 minimal promoter (Fig. 14). Our data shows that the DNA binding domain is required for transcriptional activity through the ERE2pS2Luc reporter plasmid. It can not be ruled out that environmental estrogens act through other DNA response elements such as an AP-1 site or through a raloxifene response element (39). Further experiments using reporter plasmids containing alternative response elements should answer whether the DNA binding domain is dispensable for transcriptional activation by environmental estrogens.

*Estrogen receptor with loss of hormone binding activity:* To measure the activity of the environmental estrogens on an estrogen receptor without any hormone binding activity, we introduced HE48 into MDA-MB-231 cells. The transfected cells were treated with various concentrations of estrogen, bisphenol A, and octylphenol. For every compound and every concentration tested, the amount of luciferase activity measured was about 2% (Fig. 15). Our data shows that transcriptional activation by environmental estrogens requires an intact hormone binding domain. Loss of hormone binding results in an absolute loss of transcriptional activation.

#### **4. SECONDARY OBSERVATIONS – ESTROGEN INHIBITION OF CATARACTOGENESIS**

In the course of performing the experiment on MNU-induced mammary tumors, we found that the ovariectomized rats that were not treated with hormone developed cataracts at about 5-6 months into the experiment. At the end of the experiment, the estrogen-treated animals had significantly fewer cataracts. These

observations have been compiled and submitted as a manuscript for publication. The title and authors of the manuscript are as follows: Protective Effects of Estrogen in a Rat Model of Age-related Cataracts, Robert M. Bigsby, Andrea Caperell-Grant, Horacio Cardenas.

Postmenopausal hormone therapy has been lauded as offering protection against cardiovascular disease and osteoporosis (40, 41). There is epidemiological data suggesting that estrogens are protective against cataractogenesis (42). Our observations in the rat eyes support this notion and they suggest that the MNU-treated, ovariectomized rat is a good model for studying the mechanism of this potential benefit.

The abstract of our manuscript is as follows: Women have a higher incidence of cataracts and epidemiological data suggest that this increase may be due to a lack of estrogen in postmenopausal years. We have examined the effects of estrogen on methylnitrosourea (MNU)-induced cataractogenesis in Sprague-Dawley rats. Animals were ovariectomized, injected with MNU, and treated with estradiol or estrone by a continuous-release, subcutaneous implant. In the ovariectomized control group, rats developed opaque lenses at approximately 6 months after MNU treatment. By 8 months, 74% (14/19) of ovariectomized, control rats had evident opacity in one or both eyes by simple gross inspection; 58% (22/38) of the eyes in this group were opaque. Estradiol or estrone treatment reduced the incidence of cataractous eyes to 12% or 25%, respectively. Lenses were examined under a dissecting microscope for light transmission. In the ovariectomized control group, light transmission was  $26 \pm 9.2\%$ , while lenses from estradiol-treated animals had  $72 \pm 5.8\%$  transmission. Histological examination revealed that the anterior cortex of opaque lenses was disrupted, showing hallmark signs typical of age-related cataracts; in addition, some eyes that appeared clear by macroscopic examination showed early histologic signs of cataractogenesis. Thus, the MNU-treated, ovariectomized rat serves as a model for age-related cataractogenesis and there is a clear protective effect of estrogens in this system. Using RT-PCR, it was demonstrated that lens cells express both alpha and beta types of estrogen receptor, suggesting that the protective effects of the hormones may be a direct, receptor mediated phenomenon.

## 5. RELEVANCE TO STATEMENT OF WORK

### YEAR 1, ORIGINAL SOW

- Aim 1: Begin tumor promoter experiments in MNU-initiated rats treated with test compounds. The size of this experiment mandates that it be broken into half, with all treatment groups represented in each half, i.e. the experiment will be performed in two batches. Collect and analyze blood and tissue samples for compound.
- Aim 2: Begin and complete xenograft tumor model for detection of growth promoting effects of test compounds. Begin Study of fasted vs. fed host animals.
- Aim 3: Prepare batches of cDNA from acquired plasmids and begin to construct mutants that are not available from other sources.

### PROGRESS TO DATE:

Aim 1: Since there was some concern over the positive controls, it was decided that the first order of business was to establish the tumor promoter activity of natural estrogens in the MNU-initiated rat. To that end two experiments have been completed. In the first experiment, estradiol treatment of ovariectomized animals was compared to ovariectomized control or ovary-intact control. Within 4 months it was apparent that the estradiol treatment was not replacing the tumor promoter activity supplied by the ovary; in addition, the estradiol-treated rats were not gaining weight at the rate of either of the other two groups, indicating that the dose of steroid was having a pharmacologic effect. Since high doses of estrogen had been shown to be protective against tumorigenesis in the MNU model, we decided to set up the experiment with a weaker estrogen, estrone. Both experiments were completed in the first year and their results were combined in the report. This emphasis on the positive controls has delayed the work on the xenoestrogens but they are underway and will be completed within the second year. Also, the effect of dietary restriction is being examined in the current set of experiments.

Aim 2: There has been a delay in this aim for two reasons: 1) There was a lack of personnel for the first 6 months. By the time the grant was started, Dr. Rosemary Steinmetz was no longer available to fill the technical position as originally planned; that position was filled in October, 1999. 2) We had difficulty in establishing the MCF-7 tumors using the procedures that were used in our earlier experiments. After modifications, we have established these tumors again but have only been able to complete one preliminary experiment and the positive control in this experiment did not behave to our satisfaction. The tumors are now growing in athymic hosts and the experiments, as originally designed, should be forthcoming.

Aim 3: Work on this aim has progressed so well that it is completed 1 year ahead of schedule.

### III. CONCLUSIONS

Although the standard MNU-initiated rat model would seem to be the ideal model system for doing so, the tumor promoter effects of estrogens has never been established in this system. Our initial study has shown that stimulation of mammary gland growth by estradiol or estrone is not in itself sufficient to promote tumorigenesis to same extent as the intact ovary. In addition, it appears that there is a biphasic response to estrogens: they are able to both inhibit and promote tumorigenesis and this is likely dependent upon the dose applied. Further studies are designed to define the optimum dose of estrone to promote tumorigenesis. Although it is a controversial notion (43), it may be that progesterone supplied by the ovary is also essential for full tumor promoter activity. This will be tested in experiments in which animals will be treated with estrone alone and/or with progesterone.

The studies on estrogenic effects of op-DDT and  $\beta$ -HCH in the mouse reproductive tract have produced some startling results. This is the first study in which blood levels of test compound were correlated to estrogenic response. In past reports xenoestrogens were administered in very large doses (100-500 mg/kg BW) and acute estrogen responses were monitored (4, 44-46). The impression from such studies was that xenoestrogens, being very weakly estrogenic by a number of *in vitro* assays (47-50), required the very large doses of compound to have an effect *in vivo*. In our study the compounds were delivered continuously in low doses. The blood levels attained by the highest doses in the mice were well above those which are found in the general population of people. However, even the lowest dose tested had significant biological effects in the mice and the blood levels were within one order of magnitude of blood levels found in some non-exposed humans (12). Further testing is required to determine the blood levels attained by the minimal effective dose in the animals.

The MCF-7 xenograft studies are just beginning and no conclusions can be drawn at this date. The difference in the lag for tumor generation between the techniques of tissue grafting and cell injection has been reported by others (51, 52). Also, from our experience with these and other tumors, it is likely that xenograft passaging will produce faster growing tumors that are more sensitive to hormonal stimulation.

The studies on the estrogen receptor domains which participate in estrogen and xenoestrogen transcriptional activation have given us some clues as to how compounds can produce qualitatively and quantitatively disparate effects in the same tissue. Compounds such as BPA, octylphenol, and  $\beta$ -HCH are fully effective compared to estradiol when the wild-type receptor is present; op-DDT produces a super-activation of the reporter gene. Deletion of either AF-1 or AF-2 results in a loss of relative efficacy of the xenoestrogens. Thus, they appear to require a synergy between the two activation function domains. Recent observations by Nishikawa (53) suggest that the different compounds are likely to produce different receptor - coactivator interactions. The fact that op-DDT consistently produced a greater

response than estradiol suggests that it may enhance binding of coactivator to receptor. Such differences in molecular activity of compounds should be considered when attempting to assess their potential bioactivity.

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Table 1. Summary of tumor formation and estrogenic endpoints in MNU-initiated, hormone-treated rats.

	Ovxd	Treatments		
		+E2	+E1	Intact
<b>Mammary Tumors</b>				
animals w/ tumors:	0/20 (0%) <sup>a</sup>	5/21 (24%) <sup>a</sup>	6/11 (55%) <sup>b</sup>	10/10 (100%)
tumor latency (mos):	-	4.8 ± 0.83	4.5 ± 0.55	3.4 ± 0.30
<b>Estrogenic Effects</b>				
uterine wt (mg):	132 ± 7.6 <sup>a</sup>	445 ± 33.6 <sup>b</sup>	504 ± 21.9 <sup>b</sup>	610 ± 98.4
pituitary wt (mg):	13.5 ± 0.63	22.0 ± 2.69 <sup>abc</sup>	16.0 ± 0.55 <sup>ab</sup>	11.2 ± 1.95
body wt (g):	347 ± 6.4 <sup>a</sup>	239 ± 5.2 <sup>abc</sup>	300 ± 4.9 <sup>b</sup>	298 ± 5.8

Ovxd, ovariectomized control; E2, estradiol; E1, estrone; Intact, ovary-intact control. Mammary tumor incidences were compared by chi-square analysis; all other means were compared against each other by ANOVA and Fisher's PLSD. p < 0.05: vs Intact (a); vs. Ovxd (b); vs. E1 (c)

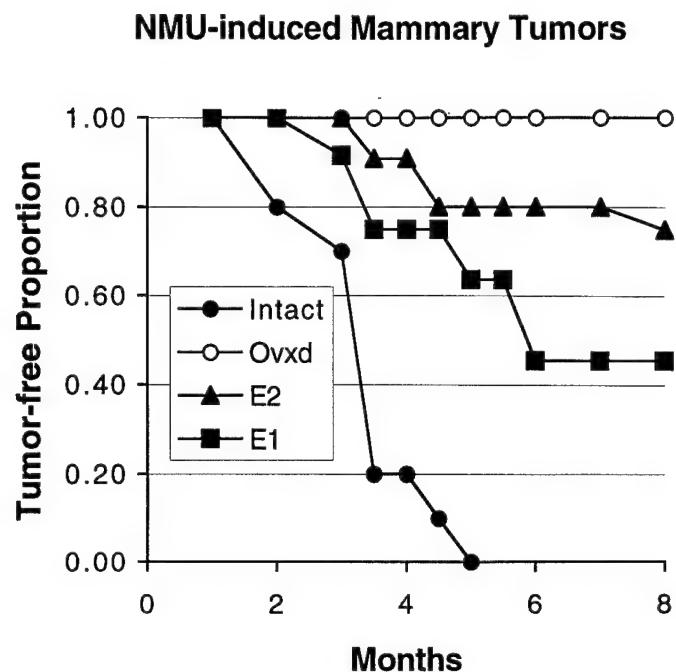


Figure 1. Time-course of tumorigenesis in MNU-treated rats. Animals were ovariectomized, injected with MNU, and implanted with a Silastic capsule containing estradiol (E2), estrone (E1), or with an empty capsule (Ovxd). An ovary-intact group served as the positive control (Intact). Animals were palpated on a weekly basis to determine the onset of tumorigenesis.

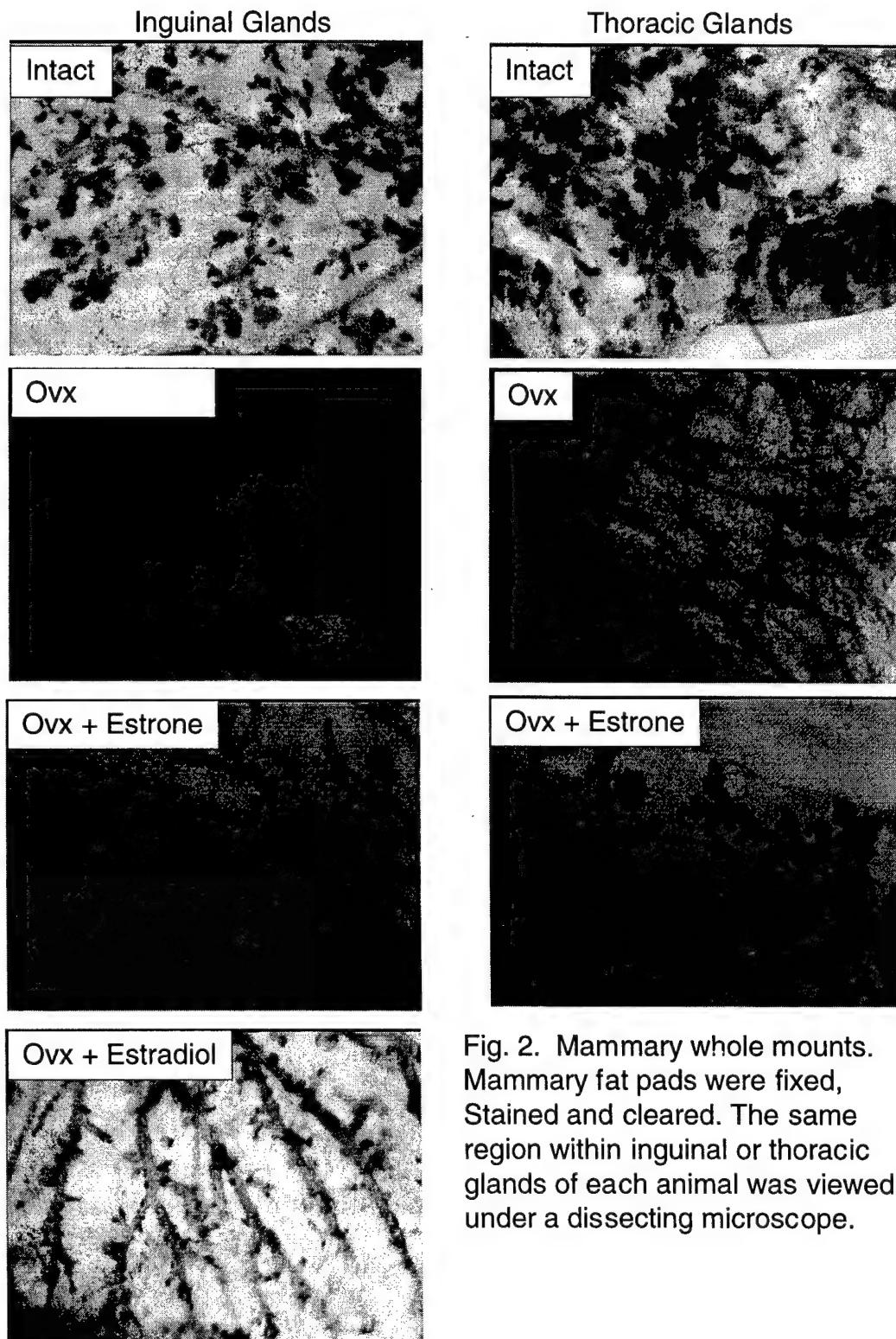
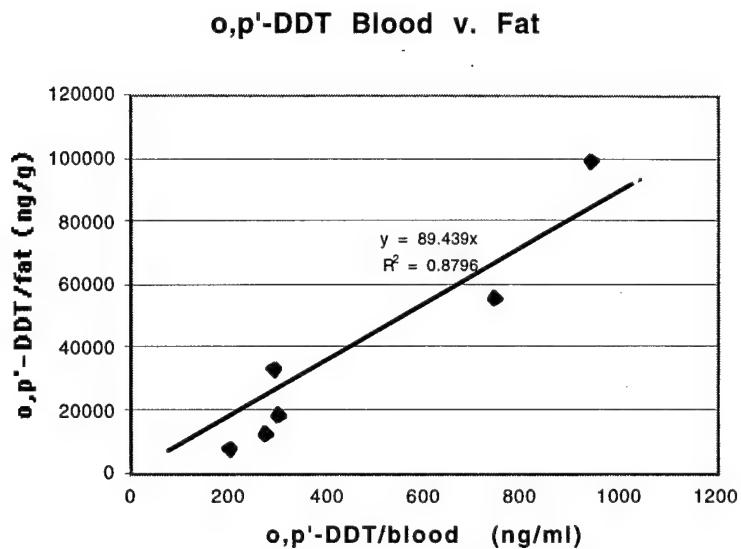


Fig. 2. Mammary whole mounts. Mammary fat pads were fixed, Stained and cleared. The same region within inguinal or thoracic glands of each animal was viewed under a dissecting microscope.

A



B

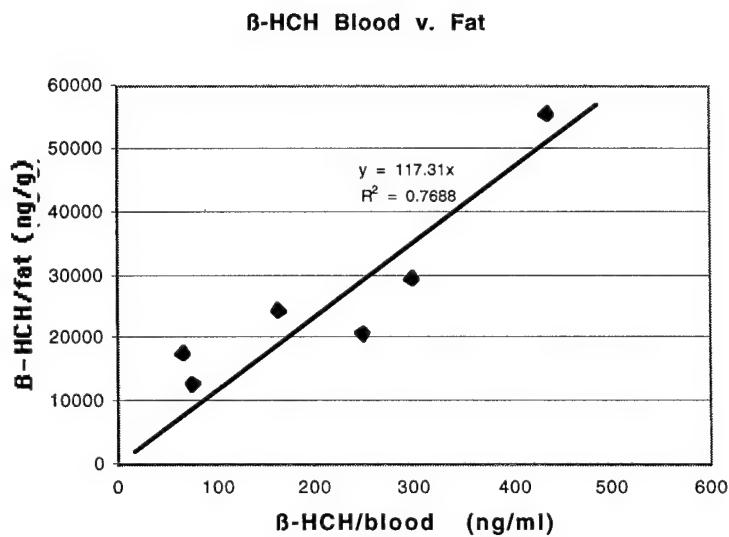


Figure 3: Blood and fat levels of o,p'-DDT &  $\beta$ -HCH following one week of treatment. One week after implanting 1, 2, or 4 Silastic capsules of o,p'-DDT or  $\beta$ -HCH animals were sacrificed; blood and corresponding fat samples were analyzed from 2 animals of each treatment group. The correlations between blood and fat levels for o,p'-DDT (A) and  $\beta$ -HCH (B) are shown.

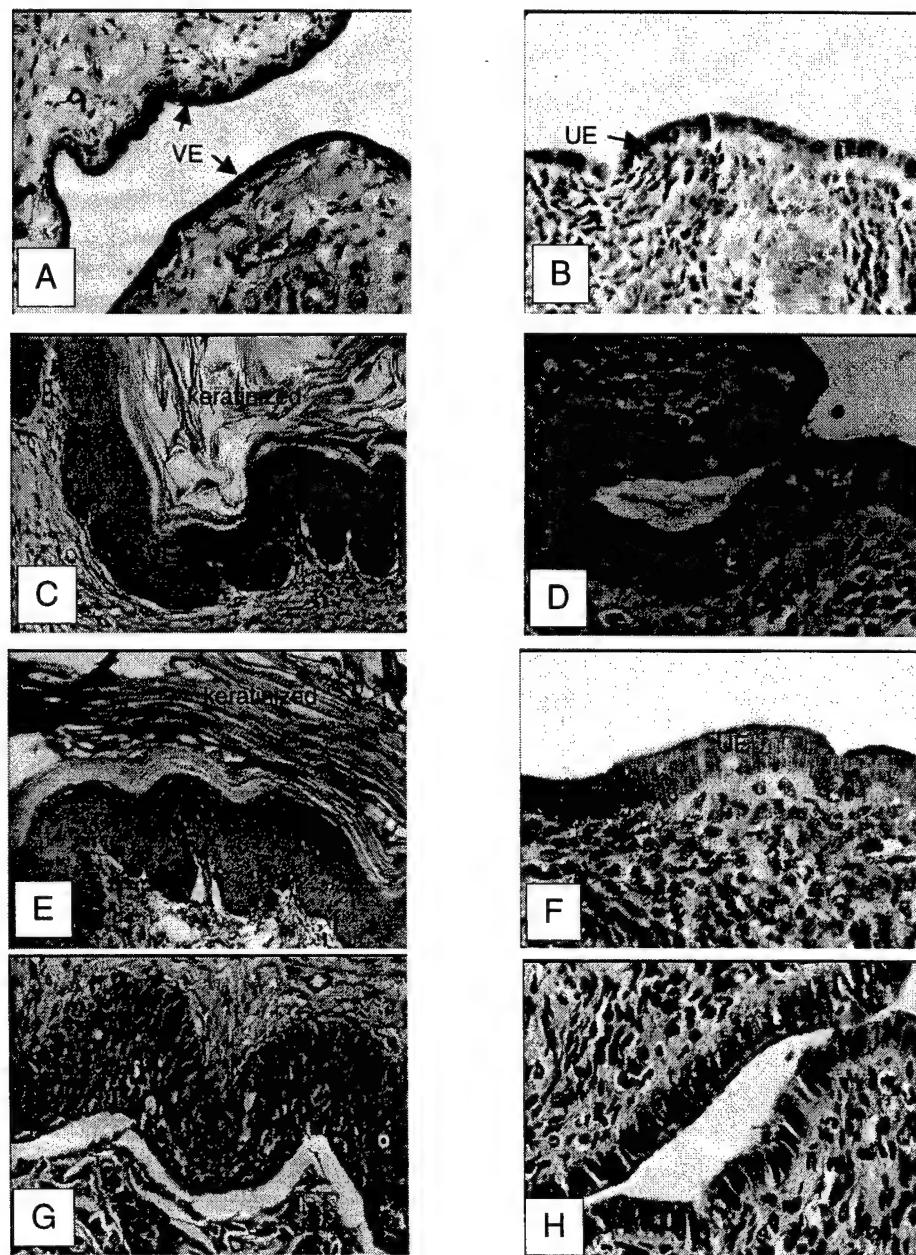


Figure 4: Histologic determination of estrogenic effects. Mice were ovariectomized and 3 weeks later they received a subcutaneous Silastic treatment implant containing either estrone (C, D), o,p'-DDT (E, F), or  $\beta$ -HCH (G, H); control animals received empty Silastic capsules (A, B). Vaginal epithelial (VE) thickness (A, C, E, G) was increased by all treatments; the superficial layer of epithelial cells became keratinized in the estrone (C) and o,p'-DDT (E) treated animals. Uterine epithelial (UE) cell height (B, D, F, H) was increased by all treatments. Objective magnification: 20X: A, G; 10X: C, E; 40X: B, D, F, H.

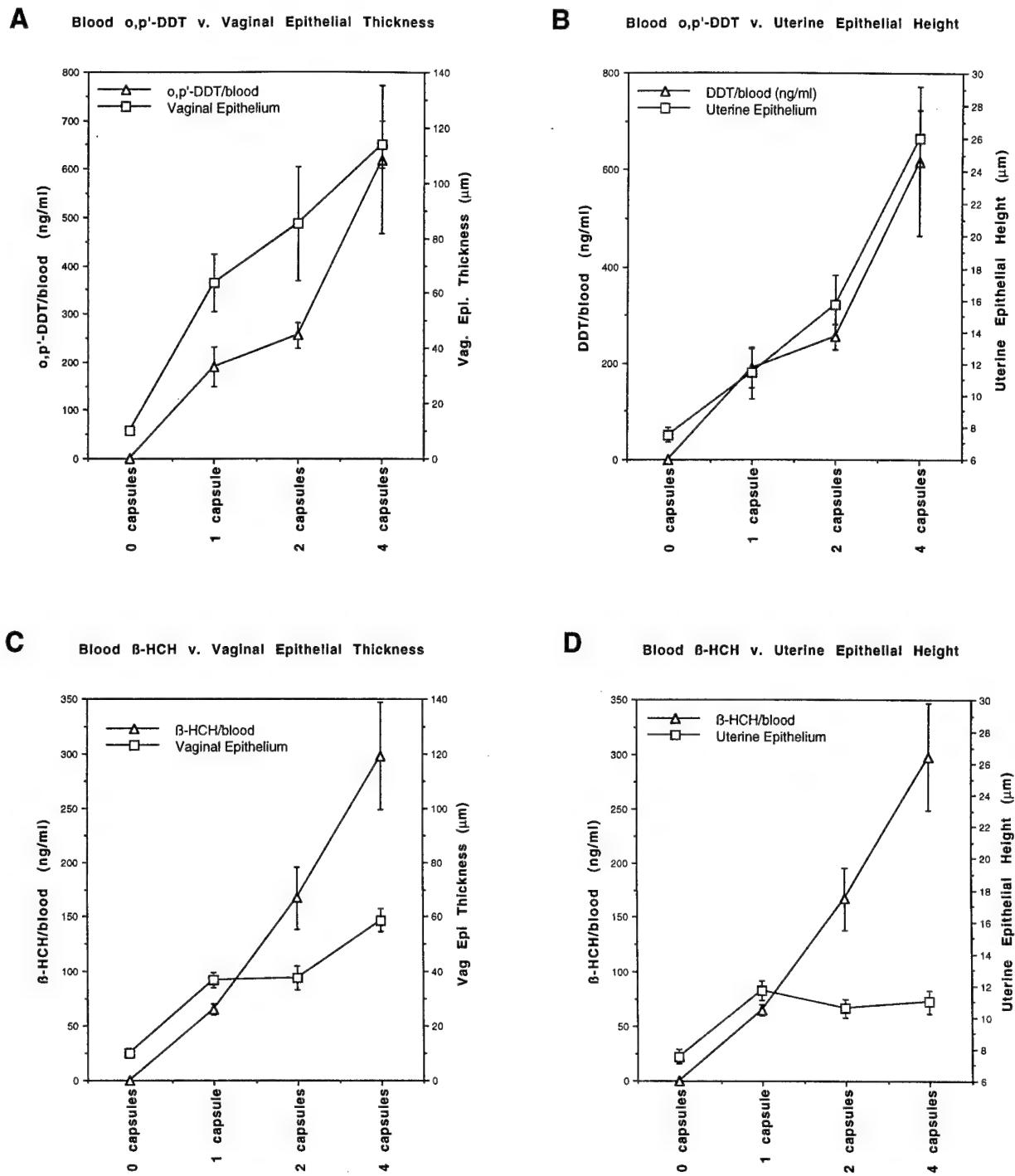


Figure 5: Dose-response curves for o,p'-DDT &  $\beta$ -HCH in mouse uterus and vagina. Vaginal epithelial thickness (A & C) or uterine epithelial cell height (B & D) were measured in animals treated for 1 week with 1, 2, or 4 capsules containing o,p'-DDT (A, B) or  $\beta$ -HCH (C, D) or with an empty capsule (0 capsules). Blood levels of test compound were analyzed in 4 animals per treatment group and histologic evaluation was done in 5 animals per group.

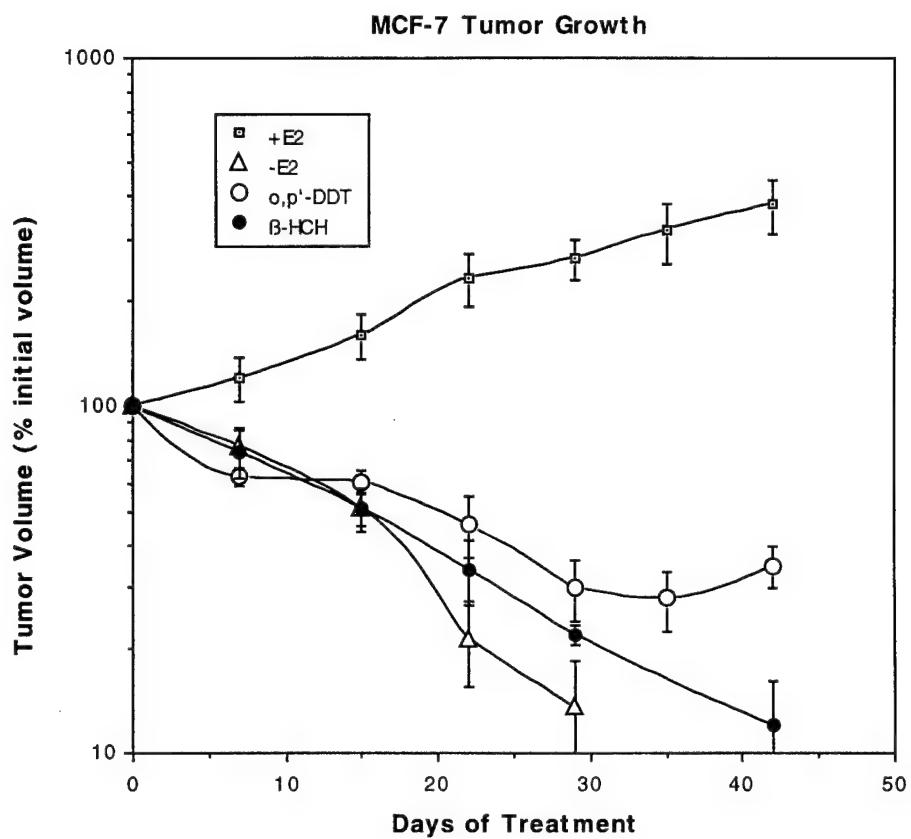
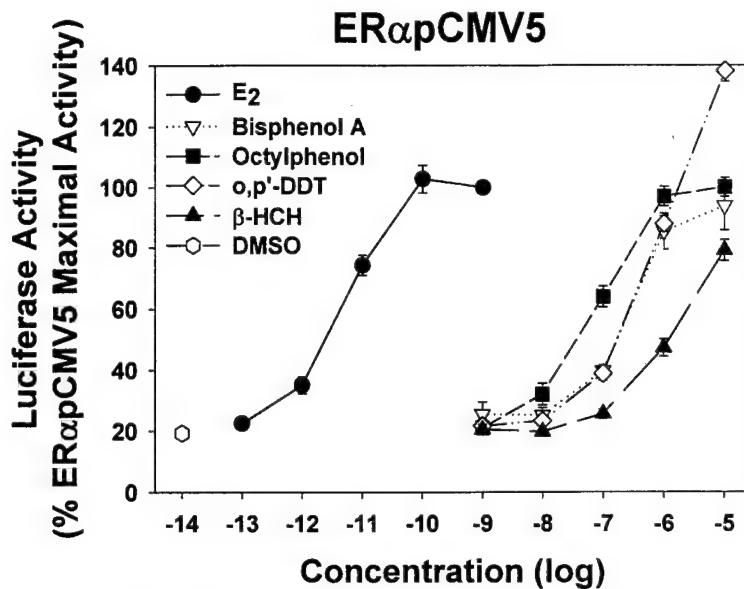
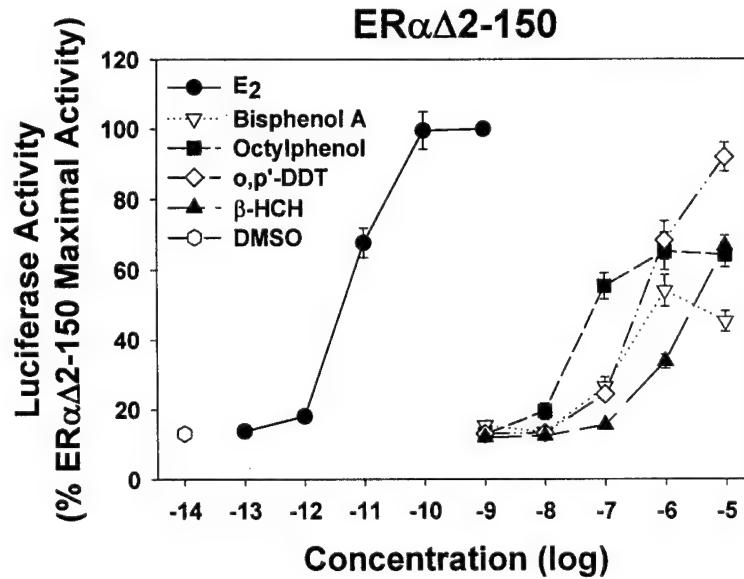


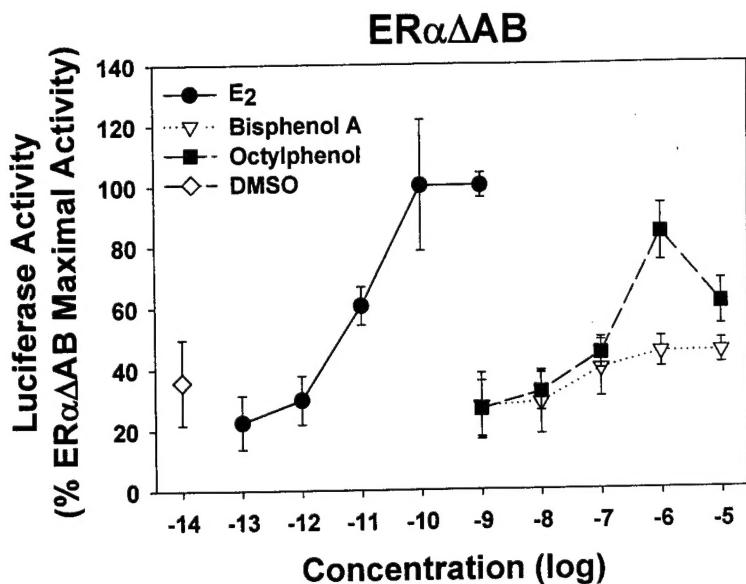
Figure 6: MCF-7 tumor growth in athymic mice. Athymic mice (16) were ovariectomized and implanted with continuous release capsules containing crystalline estradiol. MCF-7 cells were harvested from culture flasks, suspended in medium containing Matrigel, and injected into the thoracic mammary fat pads of the athymic mice. After 3 weeks the estradiol capsules were removed from 12 of the host animals and replaced with capsules containing either o,p'-DDT or  $\beta$ -HCH, or they were replaced with empty capsules (-E2). The 4 remaining mice were maintained on the original estradiol capsule treatment (+E2).



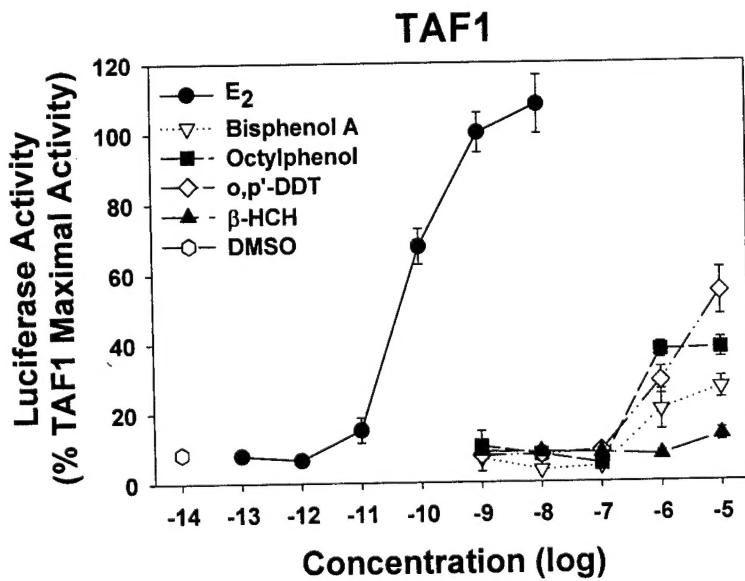
**Fig. 7. Transfection of MDA-MB-231 cells with wild-type ER $\alpha$ .** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the ER $\alpha$ pCMV5 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH, and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to the ER $\alpha$ pCMV5 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



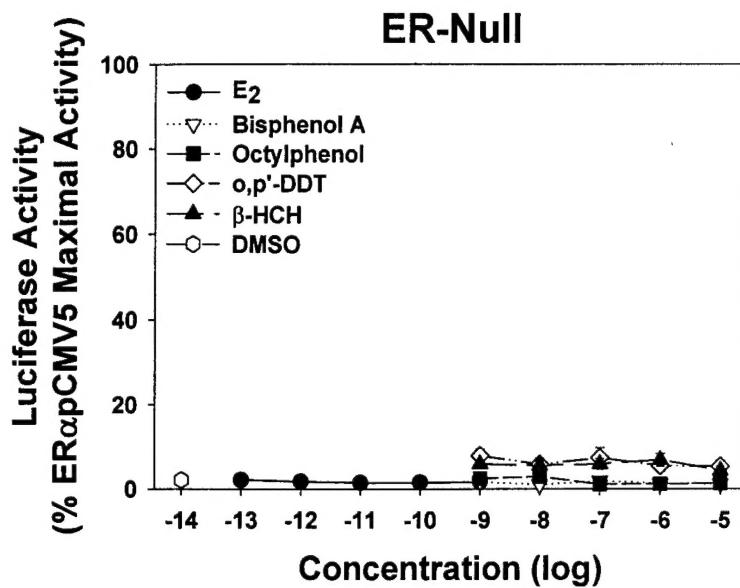
**Fig. 8. Transfection of MDA-MB-231 cells with an ER $\alpha$  mutant with loss of AF-1 function.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the ER $\alpha$  $\Delta$ 2-150 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to ER $\alpha$  $\Delta$ 2-150 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



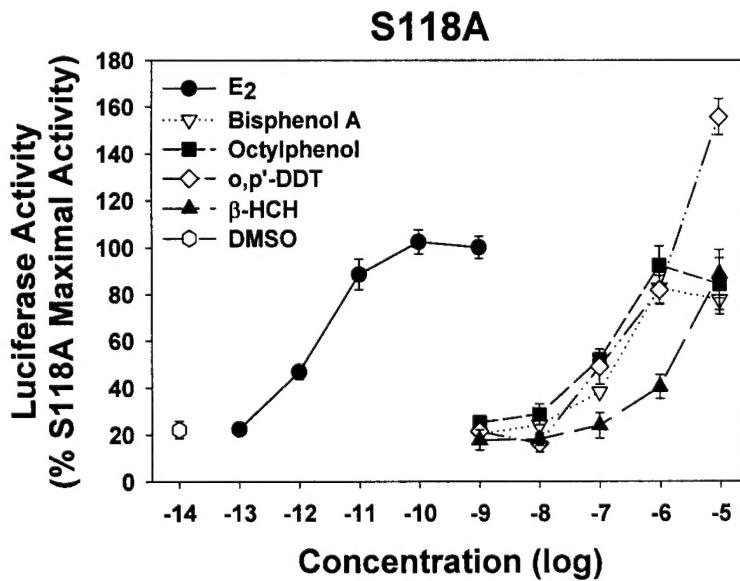
**Fig. 9. Transfection of MDA-MB-231 cells with an ER $\alpha$  mutant with loss of AF-1 function.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the ER $\alpha$ ΔAB plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to ER $\alpha$ ΔAB 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



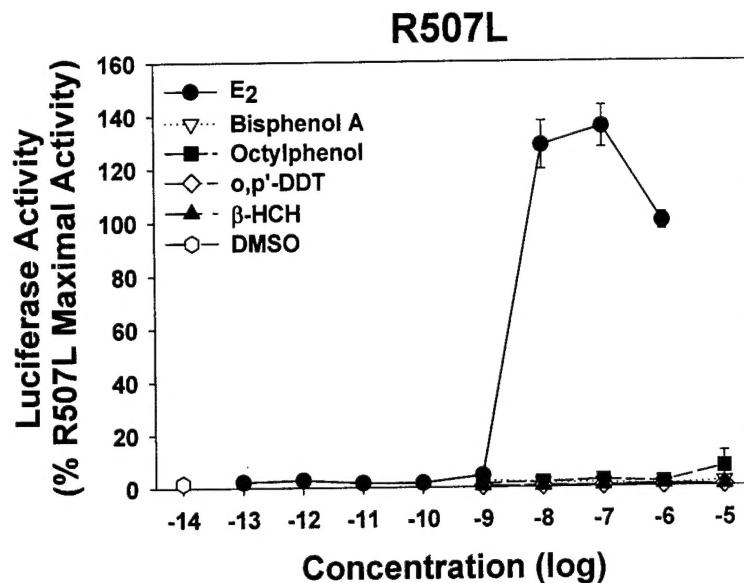
**Fig. 10. Transfection of MDA-MB-231 cells with an ER $\alpha$  mutant with loss of AF-2 function.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the TAF1 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to TAF1 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



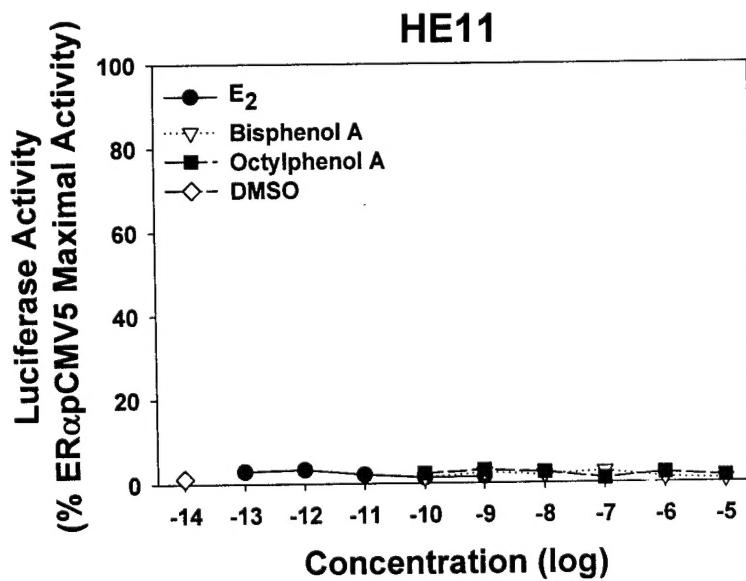
**Fig. 11. Transfection of MDA- MB-231 cells with an ER $\alpha$  mutant with loss of AF-1 and AF-2 functions.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the ER-Null plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to ER $\alpha$ pCMV5 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



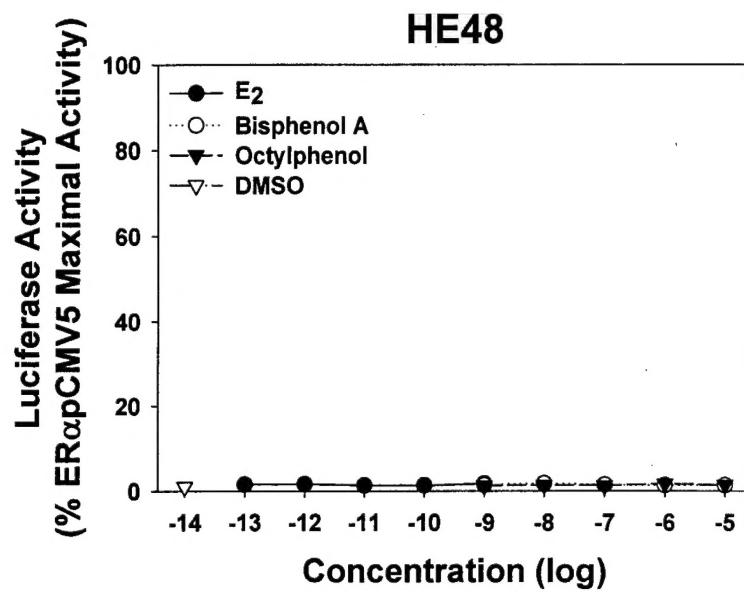
**Fig. 12. Transfection of MDA-MB-231 cells with S118A.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the S118A plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to S118A 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



**Fig. 13. Transfection of MDA-MB-231 cells with an ER $\alpha$  mutant with a reduction in binding affinity for estradiol.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the R507L plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to R507L 10<sup>-6</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



**Fig. 14. Transfection of MDA-MB-231 cells with an ER $\alpha$  mutant with loss of DNA binding activity.** ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the HE11 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to ER $\alpha$ pCMV5 10<sup>-9</sup> M E<sub>2</sub>  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to ER $\alpha$ pCMV5 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.



**Fig. 15. Transfection of MDA-MB-231 cells with ER $\alpha$  mutant with loss of hormone binding activity.**  
 ER $\alpha$  negative MDA-MB-231 cells were co-transfected with the HE48 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E<sub>2</sub>, Bisphenol A, Octylphenol, o,p'-DDT,  $\beta$ -HCH and DMSO for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity. The values represent the mean  $\pm$  SEM relative to ER $\alpha$ pCMV5 10<sup>-9</sup> M E<sub>2</sub> maximal response being 100% from three separate experiments in triplicate.